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PROCESS FOR THE HETEROTROPHIC PRODUCTION OF MICROBIAL PRODUCTS WITH HIGH CONCENTRATIONS OF OMEGA-3 HIGHLY UNSATURATED FATTY ACIDS

COMPOSITION OF MATTER AND PROCESS

This is a continuation-in-part of co-pending application serial no. 241,410, filed September 7, 1988.

BACKGROUND OF THE INVENTION

Field of the Invention

The field of this invention relates to heterotrophic organisms and a process for culturing them for the production of lipids with high concentrations of omega-3 highly unsaturated fatty acids (HUFA) suitable for human and animal consumption as food additives or for use in pharmaceutical and industrial products.

Description of the Prior Art

Omega-3 highly unsaturated fatty acids are of significant commercial interest in that they have been recently recognized as important dietary compounds for preventing arteriosclerosis and coronary heart disease, for alleviating inflammatory conditions and for retarding the growth of tumor cells. These beneficial effects are a result both of omega-3 fatty acids causing competitive inhibition of compounds produced from omega-6 fatty acids, and from beneficial compounds produced directly from the omega-3 fatty acids themselves (Simopoulos et al., 1986). Omega-6 fatty acids are the predominant highly unsaturated fatty acids found in plants and animals. Currently the only commercially available dietary source of omega-3 fatty acids is from certain fish oils which can contain up to 20-30% of these fatty acids. The beneficial effects of these fatty acids can be obtained by eating fish several times a week or by daily intake of concentrated fish oil. Consequently large quantities of fish oil are processed and encapsulated each year for sale as a dietary supplement.

However, there are several significant problems with these fish oil supplements. First, they can contain high levels of fat-soluble vitamins that are found naturally in fish oils. When ingested, these vitamins are stored and metabolized in fat in the human body rather than excreted in urine. High doses of these vitamins can be unsafe, leading to kidney problems or blindness and several U.S. medical

associations have cautioned against using capsule supplements rather than real fish. Secondly, fish oils contain up to 80% of saturated and omega-6 fatty acids, both of which can have deleterious health effects. Additionally, fish oils have a strong fishy taste and odor, and as such cannot be added to processed foods as a food additive, without negatively affecting the taste of the food product. Moreover, the isolation of pure omega-3 fatty acids from this mixture is an involved and expensive process resulting in very high prices ($200 - $1000 g) for pure forms of these fatty acids (Sigma Chemical Co., 1988; CalBiochem Co., 1987).

The natural source of omega-3 fatty acids in fish oil is algae. These highly unsaturated fatty acids are important components of photosynthetic membranes. Omega-3 fatty acids accumulate in the food chain and are eventually incorporated in fish oils. Bacteria and yeast are not able to synthesize omega-3 highly unsaturated fatty acids and only a few fungi are known which can produce minor and trace amounts of omega-3 highly unsaturated fatty acids. (Weete, 1980; Wassef, 1977; Erwin, 1973.)

Algae have been grown in outdoor cultivation ponds for the photoautotrophic production of a wide variety of products including omega-3 fatty acid containing biomass. For example, U.S. patent 4,341,038 describes a method for the photosynthetic production of oils from algae, and U.S. patent 4,615,839 describes a process for concentrating

eicosapentaenoic acid (EPA) (one of the omega-3 fatty acids) produced photosynthetically by strains of the green alga Chlorella. Photoautotrophy is the process whereby cells utilize the process of photosynthesis to construct organic compounds from CO_2 and water, while using light as an energy source. Since sunlight is the driving force for this type of production system, algal cultivation ponds require large amounts of surface area (land) to be economically viable. Due to their large size, these systems cannot be economically covered, because of high costs and technical problems, and because even transparent covers tend to block a significant amount of the sunlight. Therefore, these production systems are not axenic, and are difficult to maintain as monocultures. This is especially critical if the cultures need to be manipulated or stressed (e.g., nitrogen limited) to induce production of the desired product. Typically, it is during these periods of stress, when the cells are only producing product and are not multiplying, that contaminants can readily invade the cultures. Thus, in most cases, the biomass produced is not desirable as a food additive for human consumption without employing expensive extraction procedures to recover the lipids. Additionally, photosynthetic production of algae in outdoor systems is very costly, since cultures must be maintained at low densities (1-2g/l) to prevent light limitation of the culture. Consequently, large volumes of water must be processed to recover small quantities of algae, and since the algal cells are very tiny, expensive harvesting processes must also be employed.

Mixotrophy is an alternative mode of production whereby certain strains of algae carry on photosynthesis with light as a necessary energy source but additionally use organic compounds supplied in the medium. Higher densities can be achieved by mixotrophic production and the cultures can be maintained in closed reactors for axenic production. U.S. patents 3,444,647 and 3,316,674 describe processes for the mixotrophic production of algae. However, because of the need to supply light to the culture, production reactors of this type are very expensive to build and operate, and culture densities are still very limited.

An additional problem with the cultivation of algae for omega-3 production, is that even though omega-3 fatty acids comprise 20-40% of some strains' total fatty acids, the total fatty acid content of these algae is generally very low, ranging from 5-10% of ash-free dry weight. In order to increase the fatty acid content of the cells, they must undergo a period of nitrogen limitation which stimulates the production of lipids. However, of all the strains noted to date in the literature, and over 60 strains evaluated by the inventor, all exhibit a marked decrease in omega-3 fatty acids as a percentage of total fatty acids, when undergoing nitrogen limitation. (Erwin, 1973; Pohl and Zurheide, 1979).

With respect to economics and to utilizing omega-3 highly unsaturated fatty acids as a food additive, it would be

desirable to produce these fatty acids in a heterotrophic culture. Heterotrophy is the capacity for sustained and continuous growth and cell division in the dark in which both energy and cell carbon are obtained solely from the metabolism of an organic substrate(s). Since light does not need to be supplied to a heterotrophic culture, the cultures can be grown at very high densities in closed reactors. Heterotrophic organisms are those which obtain energy and cell carbon from organic substrates, and are able to grow in the dark. Heterotrophic conditions are those conditions that permit the growth of heterotrophic organisms, whether light is present or not. However, the vast majority of algae are predominantly photoautotrophic, and only a few types of heterotrophic algae are known. U.S. patents 3,142,135 and 3,882,635 describe processes for the heterotrophic production of protein and pigments from algae such as Chlorella, Spongiococcum, and Prototheca. However, these genera and others that have been documented to grow very well heterotrophically (e.g., Scenedesmus), do not produce omega-3 highly unsaturated fatty acids (Erwin, 1973). The very few heterotrophic algae known to produce any omega-3 fatty acids (e.g., apochlorotic diatoms or apochlorotic dinoflagellates) generally grow slowly and produce low amounts of omega-3 fatty acids as a percentage of ash-free dry weight (Harrington and Holtz, 1968; Tornabene et al., 1974).

A few higher fungi are known to produce omega-3 highly unsaturated fatty acids, but they comprise only a very small

fraction of the total fatty acids in the cells (Erwin, 1973; Wassef, 1977; Weete, 1980). As such, they would not be good candidates for commercial production of omega-3 fatty acids. For example, Yamada et al. (1987) recently reported on the cultivation of several species of the fungus, Mortierella, (isolated from soils) for the production of the omega-6 fatty acid, arachidonic acid. These fungi also produce small amounts of omega-3 eicosapentaenoic acid along with the arachidonic acid when grown at low temperatures (5-24°C). However, the resulting eicosapentaenoic acid content was only 2.6% of the dry weight of the cells, and the low temperatures necessary to stimulate production of this fatty acid in these species would result in greatly decreased productivities (and economic potential) of the cultivation system. Some single-celled members of the Fungal class Phycomycetes (algal-like fungi)... order Thraustochytriales are also known to produce omega-3 highly unsaturated fatty acids (Ellenbogen, 1969; Wassef, 1977; Weete, 1980; Findlay et al., 1986) but they are known to be difficult to culture. Sparrow (1960) noted that the minuteness and simple nature of the thalli of the family Thraustochytriaceae Class Oomycetes (order Thraustochytriales) make them exceedingly difficult to propagate. Additional reasons for this difficulty have been outlined by Emerson (1950) and summarized by Schneider (1976): "1) these fungi consist of very small thalli of only one or a few cells, which generally grow very slowly in culture, and are very sensitive to environmental perturbation; 2) they are generally saprophytes, or parasites with very specialized nutritional and environmental demands; and 3) in pure culture they generally

exhibit restricted growth, with vegetative growth terminating after a few generations." (Although some prior art classifies the thraustochytrids as fungi, the most recent consensus is that they should be classified as algae, see discussion below.)

As a result little attention has been paid to the numerous orders of these microorganisms, and those studies that have been conducted, have been predominantly carried out with a taxonomic or ecological focus. For example, even though the simple fatty acid distribution of several members of the order Thraustochytriales has been reported from a taxonomic perspective (Ellenbogen, 1969); Findlay et al., 1986), no one has ever reported their total fatty acid content or lipid content as percent dry weight. Unless data on the total lipid content is available, one cannot evaluate an organism's potential for use in the production of any type of fatty acid. For example, the omega-3 fatty acid content of the lipids of some marine macroalgae (seaweeds) is reported to be very high, up to 51% of total fatty acids (Pohl & Zurheide, 1979). However, the lipid content of macroalgae is typically very low, only 1-2% of cellular dry weight (Ryther, 1983). Therefore, despite the reported high content of omega-3 fatty acids in the fatty acids of macroalgae, they would be considered to be very poor candidate organisms for the production of omega-3 fatty acids. Despite a diligent search by the inventor, no reports of simple proximate analysis (% protein, carbohydrate and lipid) of the Thraustochytriales

has been found, nor has anyone reported attempts to cultivate these species for purposes other than laboratory studies of their taxonomy, physiology or ecology. Additionally, many of the strains of these microorganisms have been isolated by simple pollen baiting techniques (e.g., Gaertner, 1968). Pollen baiting techniques are very specific for members of the Thraustochytriales, but do not select for any characteristics which may be desirable for large scale cultivation of microorganisms.

Thus, until the present invention, there have been no known heterotrophic organisms suitable for culture that produce practical levels of omega-3 fatty acids and such organisms have been thought to be very rare in the natural environment.

BRIEF SUMMARY OF THE INVENTION

The invention provides novel organisms that are able to grow well in heterotrophic culture and yet produce practical levels of omega-3 fatty acids. Methods for selecting large numbers of such organisms from a natural environment and a medium for growing the organisms to give sustained growth and good yields of omega-3 fatty acids are also taught herein. The present invention provides a simple, bacteria-free, heterotrophic method for the production of a whole-cell product with a high concentration of omega-3 highly unsaturated fatty acids. The invention further provides a

process which produces a whole-cell product preferably with little or no pigmentation (color) and preferably having a minimal content of other saturated and omega-6 highly unsaturated fatty acids. Where desired, one can utilize thermotolerant strains to exploit rapid growth at elevated temperatures (>30°C) as well as growth at very low salinities.

In accordance with the present invention, a preferred heterotrophic method for producing microbial biomass with a high concentration of omega-3 highly unsaturated fatty acids is provided which comprises the steps of:

- collecting, isolating, and selecting heterotrophic strains of single-celled microorganisms that have high contents of omega-3 highly unsaturated fatty acids,

- growing these strains under bacteria-free, heterotrophic conditions in saline medium in the presence of an organic source of carbon and an organic or inorganic source of nitrogen,

- utilizing elevated concentrations of dissolved phosphorous and yeast extract, corn steep liquor, or other nutritional supplements containing growth factors to maintain continuously growing cells,

- preferably inducing the formation of high concentrations of lipids containing omega-3 highly unsaturated fatty acids in the cells, and

- harvesting, drying, and/or otherwise preparing the biomass for storage or further processing.

The whole-cell biomass can be used directly as a food additive to enhance the omega-3 fatty acid content and nutritional value of processed foods for human intake or for animal feed. Animals are defined as any organism belonging to the kingdom Animalia. The complex lipids containing these fatty acids can also be extracted from the whole-cell product with solvents and utilized in a more concentrated form (e.g., encapsulated) for pharmaceutical or nutritional purposes and industrial applications.

It is understood herein that a fatty acid is an aliphatic monocarboxylic acid. Lipids are understood to be fats or oils including the glyceride esters of fatty acids along with associated phosphatides, sterols, alcohols, hydrocarbons, ketones, and related compounds.

A commonly employed shorthand system is used in this specification to denote the structure of the fatty acids (e.g., Weete, 1980). This system uses the letter "C" accompanied by a number denoting the number of carbons in the

hydrocarbon chain, followed by a colon and a number indicating the number of double bonds, i.e., C20:5, eicosapentaenoic acid. Fatty acids are numbered starting at the carboxy carbon. Position of the double bonds is indicated by adding the Greek letter delta (▲) followed by the carbon number of the double bond; i.e., C20:5omega-3$\Delta^{5,8,11,14,17}$. The "omega" notation is a shorthand system for unsaturated fatty acids whereby numbering from the carboxy-terminal carbon is used. For convenience, w3 will be used to symbolize "omega-3," especially when using the numerical shorthand nomenclature described herein. Omega-3 highly unsaturated fatty acids are understood to be polyethylenic fatty acids in which the ultimate ethylenic bond is 3 carbons from and including the terminal methyl group of the fatty acid. Thus, the complete nomenclature for eicosapentaenoic acid, an omega-3 highly unsaturated fatty acid, would be C20:5w3$\Delta^{5,8,11,14,17}$. For the sake of brevity, the double bond locations ($\Delta^{5,8,11,14,17}$) will be omitted. Eicosapentaenoic acid is then designated C20:5w3, Docosapentaenoic acid (C22:5w3$\Delta^{7,10,13,16,19}$) is C22:5w3, and Docosahexaenoic acid (C22:6w3$\Delta^{4,7,10,13,16,19}$) is C22:6w3. The nomenclature "highly unsaturated fatty acid" means a fatty acid with 4 or more double bonds. "Saturated fatty acid" means a fatty acid with 1 to 3 double bonds.

NEW

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A collection and screening process was developed by the inventor to isolate readily many strains of microorganisms with the following combination of economically desirable characteristics for the production of omega-3 highly unsaturated fatty acids: 1) capable of heterotrophic growth; 2) high content of omega-3 highly unsaturated fatty acids; 3) unicellular; 4) preferably low content of saturated and omega-6 highly unsaturated fatty acids; 5) preferably nonpigmented, white or essentially colorless cells; 6) preferably thermotolerant (ability to grow at temperatures above 30°C); and 7) preferably euryhaline (able to grow over a wide range of salinities, but especially at low salinities).

Collection, isolation and selection of large numbers of suitable heterotrophic strains can be accomplished by the following method. Suitable water samples and organisms typically can be collected from shallow, saline habitats which preferably undergo a wide range of temperature and salinity variation. These habitats include marine tide pools, estuaries and inland saline ponds, springs, playas and lakes. Specific examples of these collection sites are: 1) saline warm springs such as those located along the Colorado river in Glenwood Springs, Colorado, or along the western edge of the Stansbury Mountains, Utah; 2) playas such as Goshen playa located near Goshen, Utah; 3) marine tide pools such as those located in the Bird Rocks area of La Jolla, California; and

4) estuaries, such as Tijuana estuary, San Diego County, California. Special effort should be made to include some of the living plant matter and naturally occurring detritus (decaying plant and animal matter) along with the water sample. The sample can then be refrigerated until return to the laboratory. Sampling error is minimized if the water sample is shaken for 15-30 seconds, prior to pipetting or pouring a portion, for example, 1-10 ml, into a filter unit. The filter unit includes 2 types of filters: 1) on top, a sterile Whatman #4 filter (Trademark, Whatman Inc., Clifton, N.J.); and 2) underneath the Whatman filter, a polycarbonate filter with 1.0μm pore size. The purpose of the first (top) filter is to remove all particulate matter greater than about 25μm, generally allowing only unicellular type material to pass onto the 1.0μm polycarbonate filter. The first filter greatly reduces the number of mold colonies that subsequently develop upon incubation of the polycarbonate filter, thereby enhancing the opportunities for other colonies to develop. Mold spores are very numerous in coastal and inland saline waters, and mold colonies can quickly cover an agar plate unless screened out. The 1.0μm size of the polycarbonate filter is chosen to allow many of the bacteria to pass on through into the filtrate. The purpose of using a sandwich filter design is to select for unicellular organisms at least a portion of whose cells range in diameter from about 1μm to about 25μm in size (organisms which could potentially be grown easily in a fermenter system for production on a large scale). Extensive growth of these unicellular organisms can be

encouraged by incubation of the polycarbonate filter on an agar plate. Competition between organisms growing on the filter facilitates the isolation of competitive, robust strains of single-celled microorganisms. Unicellular aquatic microorganisms selected by the foregoing method display a range of cell size depending on growth conditions and stage of reproductive cycle. Most cells in culture have diameters in the range from about 1μm to about 25μm; however, cells (thalli and sporangia) in the cultures can be found that have larger diameters (depending on the strain) up to about 60μm.

After filtration, the polycarbonate filter can be placed on an agar plate containing saline media containing a source of organic carbon such as carbohydrate including glucose, various starches, molasses, ground corn and the like, a source of assimilable organic or inorganic nitrogen such as nitrate, urea, ammonium salts, amino acids, microbial growth factors included in one or more of yeast extract, vitamins, and corn steep liquor, a source of assimilable organic or inorganic phosphorous, and a pH buffer such as bicarbonate. Microbial growth factors are currently unspecified compounds which enhance heterotrophic growth of unicellular microorganisms, including fungi and algae. The agar plates can be incubated in the dark at 25-35°C (30°C is preferred) and after 2-4 days numerous colonies will have appeared on the filter. Recovery of 1-5 colonies/plate of the desired organism is not uncommon. Yeast colonies are distinguishable either by color (they frequently are pink) or by their morphology. Yeast colonies

are smooth whereas the desired organisms form in colonies with rough or textured surfaces. Individual cells of the desired organism can be seen through a dissecting microscope at the colony borders, whereas yeast cells are not distinguishable, due to their smaller size. Clear or white colored colonies can be picked from the plates and restreaked on a new plate of similar media composition. The new plate can be incubated under similar conditions, preferably at 30°C and single colonies picked after a 2-4 day incubation period. Single colonies can then be picked and placed in, for example, 50ml of liquid medium containing the same organic enrichments (minus agar) as in the agar plates. These cultures can be incubated for 2-4 days at 30°C with aeration, for example, on a rotary shaker table (100-200 rpm.). When the cultures appear to reach maximal density, 20-40ml of the culture can then be harvested by centrifugation or other suitable method and preserved, as by lyophilization. The sample can then be analyzed by standard, well-known techniques including gas chromatography techniques to identify the fatty acid content of the strain. Those strains with omega-3 highly unsaturated fatty acids can thereby be identified and cultures of these strains maintained for further screening.

Promising strains can be screened for temperature tolerance by inoculating the strains into 250ml shaker flasks containing 50ml of culture media. These cultures are then incubated for 2 days on the shaker table over any desired temperature range from most practically between 27-48°C, one

culture at each 3°C interval. Production can be quantified as the total amount of fatty acids produced per ml of culture medium. Total fatty acids can be quantified by gas chromatography as described above. A similar process can also be employed to screen for salinity tolerance. For salinity tolerance a range of salinities yielding conductivities from 5-40 mmho/cm is adequate for most purposes. Screening for the ability to utilize a variety of carbon and nitrogen sources can also be conducted employing the procedure outlined above. The carbon and nitrogen sources were evaluated herein at concentrations of 5g/l. Carbon sources evaluated were: glucose, corn starch, ground corn, potato starch, wheat starch, and molasses. Nitrogen sources evaluated were: nitrate, urea, ammonium, amino acids, protein hydrolysate, corn steep liquor, tryptone, peptone, or casein. Other carbon and nitrogen sources can be used, the choice being open to those of ordinary skill in the art, based on criteria of significance to the user.

NEW

In a preferred process, the omega-3 HUFA's can be produced by the direct fermentation of ground, unhydrolyzed grains (e.g., corn, sorghum, rice, wheat, oats). In this process species/strains from the genus <u>Thraustochytrium</u> are utilized as the production organism. Unhydrolyzed corn syrup or agricultural/fermentation by-products such as stillage, a waste product in corn to alcohol fermentations, can also be utilized as an inexpensive carbon/nitrogen source.

In another preferred process, omega-3 HUFA's can be produced from these ground grains and waste products, if they are prehydrolyzed to sugars by an mixed hydrolysis process whereby the ground grain is first partially hydrolyzed with a mild acid treatment, followed by an enzymatic process employing an enzyme such as amylase, amyloglucosidase, alpha or beta glucosidase, or a mixture of these enzymes.

New

Using the collection and screening process outlined above, strains of unicellular fungi and algae can be isolated which have omega-3 highly unsaturated fatty acid contents up to 32% total cellular ash-free dry weight, and which exhibit growth over a temperature range from 15-48°C and grow in very low salinity culture medium.

Growth of the strains by the invention process can be effected at any temperature conducive to satisfactory growth of the strains, for example, between about 15°C and 48°C, and preferably between 25-36°C. The culture medium typically becomes more alkaline during the fermentation if pH is not controlled by acid addition or buffers. The strains will grow over a pH range from 4.0-11.0 with a preferable range of about 5.5-8.5.

When growth is carried out in large vessels and tanks, it is preferable to produce a vegetative inoculum in a nutrient broth culture by inoculating this broth culture with an aliquot from a slant culture or culture preserved at -70°C

employing the cryoprotectants dimethylsulfoxide (DMSO) or glycerol. When a young, active vegetative inoculum has then been secured, it can be transferred aseptically to larger production tanks or fermenters. The medium in which the vegetative inoculum is produced can be the same as, or different from, that utilized for the large scale production of cells, so long as a good growth of the strain is obtained.

The inventor found that single-celled strains of the order Thraustochytriales (containing omega-3 fatty acids) isolated and screened by the process outlined above, generally exhibited restricted growth, with vegetative growth terminating after a few generations as predicted by Emerson (1950) and by Schneider (1976). However, the inventor found that by maintaining relatively high concentrations of phosphorous (e.g., KH_2PO_4 > 0.2g/l) and/or adding a nutritional supplement (source of fungal growth factors) such as yeast extract or corn steep liquor (greater than 0.2g/l), continuously growing cultures of these unicellular fungi could be maintained. The ability to maintain growth for more than 2-3 generations in liquid culture is termed herein sustained growth. As a group, strains in the genus <u>Thraustochytrium</u> appear to respond more favorably to phosphate additions than those in the genus <u>Schizochytrium</u>, which appear to need less phosphate. In terms of nutritional supplements supplying fungal growth factors, corn steep liquor can be substituted for the yeast extract, and with some strains, has even a more enhanced effect for allowing the strains to achieve high

densities in culture. The corn steep liquor and yeast extract contain one or more growth factors necessary for growth of the cells. While the growth factor(s) is not presently defined, it is a component of yeast extract and corn steep liquor, and either of these well-known nutritional supplements are satisfactory. Carbon conversion efficiencies close to 50% (g cell dry weight produced/100g organic carbon added to culture medium) can easily be achieved employing this process.

A microbial product high in protein and high in omega-3 fatty acids can be produced by harvesting the cells in the exponential phase of growth. If a product significantly higher in lipids and omega-3 fatty acids is desired, the culture can be manipulated to become nitrogen limited for a suitable time, preferably in the range from 6 to 24 hours. The cultures can be transferred to a nitrogen-free medium or, preferably, the initial nitrogen content of the growth medium can be provided such that nitrogen becomes depleted late in the exponential phase. Nitrogen limitation stimulates total lipid production while maintaining high levels of omega-3 fatty acids as long as the induction period is kept short, usually 6-24 hours. Length of the induction period can be manipulated by raising or lowering temperature, depending on the strain employed. [Additionally, the cells can be cultured on a continuous basis in a medium with a high carbon-to-nitrogen ratio, enabling continuous production of high lipid content (and high omega-3 content) cellular biomass.] The unicellular strains of heterotrophic microorganisms isolated

New

by the screening procedure outlined above, tend to have high concentrations of three omega-3 highly unsaturated fatty acids: C20:5w3, C22:5w3 and C22:6w3 and very low concentrations of C20:4w6. The ratios of these fatty acids can vary depending on culture conditions and the strain employed. Ratios of C20:5w3 to C22:6w3 can run from about 1:1 to 1:30. Ratios of C22:5w3 to C22:6w3 can run from 1:12 to only trace amounts of C22:5w3. In the strains that lack C22:5w3, the C20:5w3 to C22:6w3 ratios can run from about 1:1 to 1:10. An additional highly unsaturated fatty acid, C22:5w6 is produced by some of the strains, including all of the prior art strains (up to a ratio of 1:4 with the C22:6w3 fatty acid). However, C22:5w6 fatty acid is considered undesirable as a dietary fatty acid because it can retroconvert to the C20:4w6 fatty acid. The screening procedure outlined in this patent, however, facilitates the isolation of some strains that contain no (or less than 1%) omega-6 highly unsaturated fatty acids (C20:4w6 or C22:5w6).

NEW

The concentration of naturally-occurring antioxidants in the cells, for example vitamin E and vitamin C (which protect the omega-3 HUFA's from oxidation), can also be manipulated by varying culture conditions such as temperature, salinity and nutrient concentrations. Concentrations of these antioxidants can be manipulated to increase the shelf life and stability of products made from the harvested biomass.

Other products that can be extracted from the harvested cellular biomass include: protein, carbohydrate, sterols, carotenoids, and enzymes (e.g., proteases). Strains producing high levels of omega-6 fatty acids have also been isolated. Such strains are useful for producing omega-6 fatty acids which, in turn, are useful starting materials for chemical synthesis of prostaglandins and other eicosanoids. Strains producing more than 25% of total fatty acids as omega-6 fatty acids have been isolated by the method described herein.

The harvested biomass can be dried (e.g., spray drying, tunnel drying, vacuum drying, or a similar process) and used as a feed supplement added to chicken feed to produce omega-3 HUFA enriched eggs or omega-3 HUFA enriched broiler meat. Similarly it can be added to pig feed to produce omega-3 HUFA-enriched pork, or to fish and shrimp feed to increase their omega-3 HUFA content. For most feed applications, the oil content of the harvested cells will be approximately 25-50% afdw, the remaining material being protein and carbohydrate. The protein can contribute significantly to the nutritional value of the cells as several of the strains we have evaluated have all of the essential amino acids and would be considered a nutritionally balanced protein.

In a preferred process, the freshly harvested cells (harvested by belt filtration, rotary drum filtration, centrifugation, etc.) containing omega-3 HUFA's can be mixed with a dry ground grain such as corn in order to lower the

water content of the harvested cell paste to below 40% moisture. This will allow the cell paste/corn mixture to be directly extruded, using common extrusion procedures. The extrusion temperatures and pressures can be modified to vary the degree of cell rupture in the extruded product (from all whole cells to 100% broken cells). Extrusion of the cells in this manner does not appear to greatly reduce the omega-3 HUFA content of the cells, as some of the antioxidants in the grain may help protect the fatty acids from oxidation, and the extruded matrix may also help prevent oxygen from readily reaching the fatty acids. By directly extruding the cell paste/grain mixture, drying times and costs can be greatly reduced, and it allows manipulation of the bioavailability of the omega-3 HUFA's for feed supplement applications (by degree of cell rupture).

The unicellular fungal strains isolated by the method described readily flocculate and settle, and this process can be enhanced by adjusting the pH of the culture to pH ≤ 7.0. A 6-fold concentration of the cells within 1-2 minutes can be facilitated by this process. The method can therefore be employed to preconcentrate the cells prior to harvesting, or to concentrate the cells to a very high density prior to nitrogen limitation. Nitrogen limitation (to induce higher lipid production) can therefore be carried out in a much smaller reactor, or the cells from several reactors consolidated into one reactor.

A variety of procedures can be employed in the recovery of the microbial cells from the culture medium. In a preferred recovery process, the cells produced by the subject process are recovered from the culture medium by separation by conventional means, such as by filtration or centrifugation. The cells can then be washed; frozen, lyophilized, or spray dried; and stored under a non-oxidizing atmosphere of a gas such as CO_2 or N_2 (to eliminate the presence of O_2), prior to incorporation into a processed food or feed product.

Cellular lipids containing the omega-3 highly unsaturated fatty acids can also be extracted from the microbial cells by any suitable means, such as by supercritical fluid extraction, or by extraction with solvents such as chloroform, hexane, methylene chloride, methanol, and the like, and the extract evaporated under reduced pressure to produce a sample of concentrated lipid material. The omega-3 highly unsaturated fatty acids in this preparation may be further concentrated by hydrolyzing the lipids and concentrating the highly unsaturated fraction by employing traditional methods such as urea adduction or fractional distillation (Schlenk, 1954), column chromatography (Kates, 1986), or by supercritical fluid fractionation (Hunter, 1987). The cells can also be broken or lysed and the lipids extracted into vegetable or other edible oil (Borowitzka and Borowitzka, 1988). [The extracted New oils can be refined by a well-known process for vegetable oil and used directly as a nutritional supplement feed or food

additive to produce omega-3 HUFA-enriched products. Alternatively, the oil can be further processed and purified as outlined below and then used in the above applications and also in pharmaceutical applications.

New

In a preferred process, a mixture of high purity omega-3 HUFA's or high purity HUFA's can be easily concentrated from the extracted oils. The harvested cells (fresh or dried) can be ruptured or permeabilized by well-known techniques such as sonication, liquid-shear disruption methods (e.g., French press or Manton-Gaulin homogenizer), bead milling, pressing under high pressure, freeze-thawing, freeze pressing, or enzymatic digestion of the cell wall. The lipids from the ruptured cells are extracted by use of a solvent or mixture of solvents such as such as hexane, chloroform, ether, or methanol. The solvent is removed (for example by a vacuum rotary evaporator, which allows the solvent to be recovered and reused) and the lipids hydrolyzed by using any of the well-known methods for converting triglycerides to free fatty acids or esters of fatty acids including base hydrolysis, acid hydrolysis, or enzymatic hydrolysis. The hydrolysis should be carried out a as low a temperature as possible (e.g., room temperature to 60°C) and under nitrogen to minimize breakdown of the omega-3 HUFA's. After hydrolysis is completed, the nonsaponifiable compounds are extracted into a solvent such as ether, hexane or chloroform and removed. The remaining solution is then acidified by addition of an acid such as HCl, and the free fatty acids extracted into a solvent such as

New

hexane, ether, or chloroform. The solvent solution containing the free fatty acids can then be cooled to -60 to -74°C and the non-HUFA's allowed to crystallize. The crystallized fatty acids (saturated fatty acids, and mono-, di-, and tri-enoic fatty acids) can then be removed (while keeping the solution at -60 to -74°C) by filtration, centrifugation or settling. The HUFA's remain dissolved in the filtrate (or supernatant). The solvent in the filtrate (or supernatant) can then be removed leaving a mixture of fatty acids which are >90% purity in either omega-3 HUFA's or HUFA's which are greater than or equal to 20 carbons in length. The purified omega-3 highly unsaturated fatty acids can then be used as a nutritional supplement for humans, as a food additive, or for pharmaceutical applications. For these uses the purified fatty acids can be encapsulated or used directly. Antioxidants can be added to the fatty acids to improve their stability.

NEW

The advantage of this process is that it is not necessary to go through the urea complex process to remove saturated and mono-unsaturated fatty acids prior to cold crystallization. This advantage is enabled by starting the purification process with an oil consisting of a simple fatty acid profile such as that produced by thraustochytrids (3 or 4 saturated or monounsaturated fatty acids and 3 or 4 HUFA's, two groups of fatty acids widely separated in terms of their crystallization temperatures) rather than a complex oil such as fish oil with up to 20 fatty acids (representing a

continuous range of saturated, mono-, di-, tri-, and polyenoic fatty acids, and as such an series of overlapping crystallization temperatures).

NEW

In a preferred process, the omega-3 enriched oils can be produced through cultivation of strains of the genus Thraustochytrium. After the oils are extracted from the cells by any of several well-known methods, the remaining extracted (lipids removed) biomass which is comprised mainly of proteins and carbohydrates, can be sterilized and returned to the fermenter, where the strains of Thraustochytrium can directly recycle it as a nutrient source (source of carbon and nitrogen). No prehydrolysis or predigestion of the cellular biomass is necessary. Extracted biomass of the genus Schizochytrium can be recycled in a similar manner if it is first digested by an acid and/or enzymatic treatment.

NEW

The present invention will be described in more detail by way of working examples. Species meeting the selection criteria described above have not been described in the prior art. By employing these selection criteria, the inventor isolated over 25 potentially promising strains from approximately 1000 samples screened. Out of the approximate 20,500 strains in the American Type Culture Collection (ATCC), 10 strains were later identified as belonging to the same taxonomic group as the strains isolated by the inventor. Those strains still viable in the Collection were procured and used to compare with strains isolated and cultured by the

disclosed procedures. The results of this comparison are presented in Examples 5 and 6 below.

NEW

Since the filing of the parent case, recent developments have resulted in revision of the taxonomy of the Thraustochytrids. The most recent taxonomic theorists place them with the algae. However, because of the continued taxonomic uncertainty, it would be best for the purposes of the present invention to consider the strains as Thraustochydrids (Order: Thraustochytriales; Family: Thraustochytriaceae; Genus: Thraustochytrium or Schizochytrium). The most recent taxonomic changes are summarized below:

NEW

All of the strains of unicellular microorganisms disclosed and claimed herein are members of the order OOmycetes Thraustochytriales. Thraustochytrids are marine eukaryotes with a rocky taxonomic history. Problems with the taxonomic placement of the Thraustochytrids have been reviewed most recently by Moss (1986), Bahnweb and Jackle (1986) and Chamberlain and Moss (1988). For convenience purposes, the Thraustochytrids were first placed by taxonomists with other colorless zoosporic eukaryotes in the Phycomycetes (algae-like fungi). The name Phycomycetes, however, was eventually dropped from taxonomic status, and the thraustochytrids retained in the Oomycetes (the biflagellate zoosporic fungi). It was initially assumed that the Oomycetes were related to the heterokont algae, and eventually a wide range of

ultrastructural and biochemical studies, summarized by Barr (1983) supported this assumption. The Oomycetes were in fact accepted by Leedale (1974) and other phycologists as part of the heteroknot algae. However, as a matter of convenience resulting from their heterotrophic nature, the Oomycetes and thraustochytrids have been largely studied by mycologists (scientists who study fungi) rather than phycologists (scientists who study algae).

From another taxonomic perspective, evolutionary biologists have developed two general schools of thought as to how eukaryotes evolved. One theory proposes an exogenous origin of membrane-bound organelles through a series of endosymbioses (Margulis (1970); e.g., mitochondria were derived from bacterial endosymbionts, chloroplasts from cyanophytes, and flagella from spirochaetes). The other theory suggests a gradual evolution of the membrane-bound organelles from the non-membrane-bounded systems of the prokaryote ancestor via an autogenous process (Cavalier-Smith 1975). Both groups of evolutionary biologists however, have removed the Oomycetes and thraustochytrids from the fungi and place them either with the chromophyte algae in the kingdom Chromophyta (Cavalier-Smith 1981) or with all algae in the kingdom Protoctista (Margulis and Sagan (1985).

With the development of electron microscopy, studies on the ultrastructure of the zoospores of two genera of Thraustochytrids, <u>Thraustochytrium</u> and <u>Schizochytrium</u>,

(Perkins 1976; Kazama 1980; Barr 1981) have provided good evidence that the Thraustochytriaceae are only distantly related to the Oomycetes. Additionally, more recent genetic data representing a correspondence analysis (a form of multivariate statistics) of 5S ribosomal RNA sequences indicate that the Thraustochytriales are clearly a unique group of eukaryotes, completely separate from the fungi, and most closely related to the red and brown algae, and to members of the Oomycetes (Mannella et al. 1987). Recently however, most taxonomists have agreed to remove the Thraustochytrids from the Oomycetes (Bartnicki-Garcia 1988).

In summary, employing the taxonomic system of Cavalier-Smith (1981, 1983), the Thraustochytrids are classified with the chromophyte algae in the kingdom Chromophyta, one of the four plant kingdoms. This places them in a completely different kingdom from the fungi, which are all placed in the kingdom Eufungi. The taxonomic placement of the Thraustochytrids is therefore summarized below:

Kingdom:	Chromophyta
Phylum:	Heterokonta
Order:	Thraustochytriales
Family:	Thraustochytriaceae
Genus:	Thraustochytrium or Schizochytrium

Despite the uncertainty of taxonomic placement within higher classifications of Phylum and Kingdom, the

Thraustochytrids remain a distinctive and characteristic grouping whose members remain classifiable within order Thraustochytriales.

Omega-3 highly unsaturated fatty acids are nutritionally important fatty acids for both humans and animals. Currently the only commercially available source of these fatty acids is from fish oil. However, there are several significant problems with the use of fish oil as a food or feed additive or supplement. First and most significantly, fish oils have a strong fishy taste and odor, and as such cannot be added to processed foods as a food additive, without negatively affecting the taste of the food product. This is also true for many of its applications as a feed additive. For example, experiments by the inventor and others have indicated that laying hens readily go off their feed when fed for more than a few days on feed enriched with fish oils. Fish oils are very unstable, easily becoming rancid and thereby decreasing the palatability and nutritional value of feed.

Secondly, fish oils generally only contain 20-30% omega-3 HUFA's. Desirable omega-3 HUFA contents in marine larval fish and shrimp feeds can be as high as 5-10% of their dry weight. To constitute an appropriate synthetic diet containing 5-10% omega-3 HUFA could require a diet of 15-30% fish oil. Such a synthetic diet would not be the most suitable for these larval organisms either in terms of palatability, digestibility, or stability (Sargent et al.

(1989). In terms of human nutrition, the other 70-80% of fatty acids in fish oil are saturated and omega-6 fatty acids, fatty acids which can have deleterious health benefits for humans. Processes for the isolation of pure omega-3 fatty acids from fish oils are involved and expensive, resulting in very high prices ($200-$1000 g) for pure forms of these fatty acids, much too expensive for use as a food or feed additive (Sigma Chemical Co., 1988; CalBiochem Co., 1988).

Third, most feeds currently used by the aquaculture industry are grain based feeds, and as such, are relatively low in omega-3 content. Recent surveys of seafood products have demonstrated that fish and shrimp produced by aquaculture farms generally only have 1/3-1/2 the omega-3 HUFA content of wild caught fish and shrimp (Pigott 1989). For aquacultured organisms, many which are prized because of their mild, non-fishy taste, increasing the fish oil content of their food is not effective, because it results in a fish tasting product.

As a result of the problems described above, there is an important need for development of alternative (non-fish based) sources of omega-3 HUFA's.

The microbial product of the present invention can be used as a feed supplement to provide an improved source of omega-3 fatty acids which has significant advantages over conventional sources. Poultry fed a diet supplemented with the microbial product incorporate the omega-3 fatty acids into

body tissues and into eggs. The eggs exhibit no fishy odor or taste, no change in yolk color. The poultry do not stop eating the supplemented feed, as they do with fish oil-supplemented feed. Feed supplemented with the microbial product of the present invention has a normal shelf life and does not become rancid upon standing at room temperature for several days. The eggs and flesh of poultry fed according to the invention are useful in human nutrition as sources of omega-3 fatty acids, yet are low in omega-6 fatty acid content and lack a fishy flavor.

The microbial product of the present invention is also of value as a source of omega-3 fatty acids for fish, shrimp and other products produced by aquaculture. The product can be added directly as a supplement to the feed or it can be fed to brine shrimp or other live feed organism intended for consumption by the aquacultured product. The use of such supplement enables the fish or shrimp farmer to bring to market an improved product retaining the taste advantages provided by aquaculture but having the high omega-3 content of wild caught fish coupled to the additional health advantage of reduced omega-6 fatty acid content.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a bar graph showing the effects of various media supplements on fatty acid yield, using Thraustochytrium sp. UT42-2 (ATCC No. 20891), a strain isolated according to

the selection method of the invention as test strain. The experimental procedure is described in Example 2. Ordinate: fatty acid yield, normalized to control, FFM media without supplements. Abscissa: specific additions, 1) 2x "B"-vitamin mix; 2) 2x "A" vitamin mix; 3) 2x PI metals; 4) 28mg/l KH_2PO_4; 5) treatments 2), 3) and 4) combined; and 6) 480mg/l KH_2PO_4.

Figure 2 is a graphical representation of highly unsaturated fatty acid production in newly isolated strains of the invention, represented by ■, and previously isolated strains represented by +. Each point represents a strain, the position of each point is determined by the percent by weight of total fatty acids which were omega-3 fatty acids (abscissa) and the percent by weight of total fatty acids which were omega-6 fatty acids (ordinate). Only those strains of the invention were plotted wherein less than 10.6% (w/w) of total fatty acids were omega-6 and more than 67% of total fatty acids were omega-3. Data from Table 4.

Figure 3 is a graphical representation of highly unsaturated fatty acid production in newly isolated strains of the invention, represented by ■, and previously isolated strains, represented by +. Each point represents a strain, the position of each point is determined by the percent by weight of total fatty acids which were omega-3 fatty acids (abscissa) and percent of weight of total fatty acids which were eicosapentaenoic acid (EPA C20:5w3) (ordinate). Only those strains of the invention were plotted wherein more than

67% (w/w) of total fatty acids were omega-3 and more than 7.8% (w/w) of total fatty acids were C20:5w3.

Figure 4 is a graphical representation of omega-3 fatty acid composition in newly isolated strains of the invention, represented by □, and previously isolated strains, represented by +. Each point represents a separate strain. Values on the abscissa are weight fraction of total omega-3 fatty acids which were C20:5w3 and on the ordinate are weight fraction of total omega-3 fatty acids which were C22:6w3. Only strains of the invention were plotted having either a weight fraction of C20:5w3 28% or greater, or a weight fraction of C22:6w3 greater than 93.6%.

Figure 5 is a graph showing growth of various newly isolated strains of the invention and previously isolated strains, at 25°C and at 30°C. Growth rates are normalized to the growth rate of strain U-30 at 25°C. Previously isolated strains are designated by their ATCC accession numbers. Numerical data in terms of cell number doublings per day are given in Table 5.

Figure 6 is a graph of total yields of cellular production after induction by nitrogen limitation. Each of ash-free dry weight, total fatty acids and omega-3 fatty acids, as indicated, was plotted, normalized to the corresponding value for strain 28211. All strains are identified by ATCC accession numbers.

Figure 7 is a graph of fatty acid yields after growth in culture media having the salinity indicated on the abscissa. Strains shown are newly isolated strains S31 (ATCC 20888) (□) and U42-2 (ATCC 20891) (+) and previously isolated strains, ATCC 28211 (◊) and ATCC 28209 (Δ). Fatty acid yields are plotted as relative yields normalized to an arbitrary value of 1.00 based on the average growth rate exhibited by S31 (ATCC 20888) (□) over the tested salinity range.

New

Figure 8 is a graph of increases in the omega-3 highly unsaturated fatty acid content of the total lipids in the brine shrimp, <u>Artemia</u> <u>salina</u>, fed Thraustochytrid strain (ATCC 20890) isolated by the method in Example 1. EPA = C20:5w3; DHA = C22:5w3.

Figure 9 is a graph of increases in the omega-3 highly unsaturated fatty acid content of the total lipids in the brine shrimp, <u>Artemia</u> <u>salina</u>, fed thraustochytrid strain (ATCC 20888) isolated by the method in Example 1. EPA = C20:5w3; DHA = C22:5w3.

EXAMPLES

Example 1. Collection and Screening

A 150ml water sample was collected from a shallow, inland saline pond and stored in a sterile polyethylene bottle. Special effort was made to include some of the living plant material and naturally occurring detritus (decaying plant and animal matter) along with the water sample. The sample was placed on ice until return to the laboratory. In the lab, the water sample was shaken for 15-30 seconds, and 1-10ml of the sample was pipetted or poured into a filter unit containing 2 types of filters: 1) on top, a sterile 47mm diameter Whatman #4 filter having a pore size about 25μm; and 2) underneath the Whatman filter, a 47mm diameter polycarbonate filter with about 1.0μm pore size. Given slight variations of nominal pore sizes for the filters, the cells collected on the polycarbonate filter range in size from about 1.0μm to about 25μm.

The Whatman filter was removed and discarded. The polycarbonate filter was placed on solid F-1 media in a petri plate, said media consisting of (per liter): 600ml seawater (artificial seawater can be used), 400ml distilled water, 10g agar, 1g glucose, 1g protein hydrolysate, 0.2g yeast extract, 2ml 0.1 M KH_2PO_4, 1ml of a vitamin solution (A-vits) (Containing 100mg/l thiamine, 0.5mg/l biotin, and 0.5mg/l cyanocobalamin), 5ml of a trace metal mixture (PII metals, containing per liter: 6.0g Na_2EDTA, 0.29g $FeCl_3 \cdot 6H_2O$, 6.84g

H_3BO_3, 0.86 $MnCl_2 \cdot 4H_2O$, 0.06g $ZnCl_2$, 0.026g $CoCl_2 \cdot 6H_2O$, 0.052g $NiSO_4 \cdot H_2O$, 0.002g $CuSo_4 \cdot 5H_2O$, and 0.005g $Na_2MoO_4 \cdot 2H_2O$), and 500mg each of streptomycin sulfate and penicillin-G. The agar plate was incubated in the dark at 30°C. After 2-4 days numerous colonies appeared on the filter. Colonies of unicellular fungi (except yeast) were picked from the plate and restreaked on a new plate of similar media composition. Special attention was made to pick all colonies consisting of colorless or white cells. The new plate was incubated at 30°C and single colonies picked after a 2-4 day incubation period. Single colonies were then picked and placed in 50ml of liquid medium containing the same organic enrichments as in the agar plates. These cultures were incubated for 2-4 days at 30°C on a rotary shaker table (100-200 rpm). When the cultures appeared to reach maximal density, 20-40ml of the culture was harvested, centrifuged and lyophilized. The sample was then analyzed by standard, well-known gas chromatographic techniques (e.g., Lepage and Roy, 1984) to identify the fatty acid content of the strain. Those strains with omega-3 highly unsaturated fatty acids were thereby identified, and cultures of these strains were maintained for further screening.

Using the collection and screening process outlined above, over 150 strains of unicellular fungi have been isolated which have omega-3 highly unsaturated fatty acid contents up to 32% total cellular ash-free dry weight, and which exhibit growth over a temperature range from 15-48°C.

Strains can also be isolated which have less than 1% (as % of total fatty acids) of the undesirable C20:4w6 and C22:5w6 highly unsaturated fatty acids. Strains of these fungi can be repeatedly isolated from the same location using the procedure outlined above. A few of the newly isolated strains have very similar fatty acid profiles. The possibility that some are duplicate isolates of the same strain cannot be ruled out at present. Further screening for other desirable traits such as salinity tolerance or ability to use a variety of carbon and nitrogen sources can then be carried out using a similar process.

Example 2. Maintaining unrestricted cell growth: phosphorus

Cells of Thraustochytrium sp. U42-2 (ATCC No. 20891), a strain isolated by the method in Example 1, were picked from solid F-medium and inoculated into 50ml of modified FFM medium (Fuller et al., 1964). This medium containing: seawater, 1000ml; glucose, 1.0g; gelatin hydrolysate, 1.0g; liver extract, 0.01g; yeast extract, 0.1g; PII metals, 5ml; 1ml B-vitamins solution (Goldstein et al., 1969); and 1ml of an antibiotic solution (25g/l streptomycin sulfate and penicillin-G). 1.0ml of the vitamin mix (pH 7.2) contains: thiamine HCl, 200μg; biotin, 0.5μg; cyanocobalamin, 0.05μg; nicotinic acid, 100μg; calcium pantothenate, 100μg; riboflavin, 5.0μg; pyridoxine HCl, 40.0μg; pyridoxamine·2HCl, 20.0μg; p-aminobenzoic acid, 10μg; chlorine HCl, 500μg; inositol, 1.0mg; thymine, 0.8mg; orotic acid, 0.26mg; folinic

acid, 0.2μg; and folic acid, 2.5μg. 250ml erlenmeyer flasks with 50ml of this medium were placed on an orbital shaker (200 rpm) at 27°C for 2-4 days, at which time the culture had reached their highest densities. One ml of this culture was transferred to a new flask of modified FFM medium, with the extra addition of one of the following treatments on a per liter basis: 1) 1ml of the B-vitamin mix; 2) 1ml of A-vitamin solution; 3) 5ml PII Metals; 4) 2ml of 0.1 M KH_2PO_4 (≈28mg); 5) treatments 2, 3, and 4 combined; and 6) 480mg KH_2PO_4. One ml of the culture was also transferred to a flask of modified FFM medium which had no extra additions made to it and served as a control for the experiment. The cultures were incubated for 48 hr. at 27°C on a rotary shaker (200 rpm). The cells were then harvested by centrifugation and the fatty acids were quantified by gas chromatography. The results are illustrated in Figure 1 and Table 1. In Figure 1, the yields are plotted as ratios of the control, whose relative yield is therefore 1.0. Treatments 1-6 are as follows: 1) 2x concentration of B vitamins; 2) 2x concentration of A vitamins; 3) 2x concentration of trace metals; 4) 2x concentration of (B vitamins + phosphate + trace metals); 5) 2x concentration of phosphate; and 6) 24 mg phosphate per 50ml (.48g per liter). Only the treatment of adding 0.48g KH_2PO_4 per liter resulted in enhanced growth and resulted in significantly increased fatty acid yield.

Table 1. Effect of various nutrient additions on the yield of fatty acids in <u>Thraustochytrium</u> sp. U42-2 (ATCC No. 20891)

Treatment	Fatty Acid Yield mg/liter
Control	23
2x concentration B vitamin mix	17
2x concentration A vitamin mix	24
2x concentration trace metals	27
2x concentration B vitamin mix, 2x PO_4 and 2x concentration trace metals	24
2x concentration PO_4	23
24mg phosphate per 50 ml	45

Example 3. Maintaining unrestricted growth: PO_4 and yeast extract

Cells of Schizochytrium aggregatum (ATCC 28209) were picked from solid F-1 medium and inoculated into 50ml of FFM medium. The culture was placed on a rotary shaker (200 rpm) at 27°C. After 3-4 days, 1ml of this culture was transferred to 50ml of each of the following treatments: 1) FFM medium (as control); and 2) FFM medium with the addition of 250mg/l KH_2PO_4 and 250mg/l yeast extract. These cultures were placed on a rotary shaker (200 rpm) at 27°C for 48 hr. The cells were harvested and the yield of cells quantified. In treatment 1, the final concentration of cells on an ash-free dry weight basis was 616mg/l. In treatment 2, the final concentration of cells was 1675mg/l, demonstrating the enhanced effect of increasing PO_4 and yeast extract concentrations in the culture medium.

Example 4. Maintaining unrestricted growth: substitution of corn steep liquor for yeast extract

Cells of Schizochytrium sp. S31 (ATCC No. 20888) were picked from solid F-1 medium and placed into 50ml of M-5 medium. This medium consists of (on a per liter basis): NaCl, 25g; $MgSO_4 \cdot 7H_2O$, 5g; KCl, 1g; $CaCl_2$, 200mg; glucose, 5g; glutamate, 5g; KH_2PO_4, 1g; PII metals, 5ml; A-vitamins solution, 1ml; and antibiotic solution, 1ml. The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. Sterile solutions of corn steep liquor (4g/40ml;

pH 7.0) and yeast extract 1g/40ml; pH 7.0) were prepared. To one set of M-5 medium flasks, the following amount of yeast extract solution was added: 1) 2ml; 2) 1.5ml; 3) 1ml; 4) 0.5ml; and 5) 0.25ml. To another set of M-5 medium flasks the yeast extract and corn steep liquor solutions were added at the following levels: 1) 2ml yeast extract; 2) 1.5ml yeast extract and 0.5ml corn steep liquor; 3) 1.0ml yeast extract and 1.0ml corn steep liquor; 4) 0.5ml yeast extract and 1.5ml corn steep liquor; and 5) 2ml corn steep liquor. One ml of the culture in F-1 medium was used to inoculate each flask. They were placed on a rotary shaker at 27°C for 48 hr. The cells were harvested by centrifugation and the yield of cells (as ash-free dry weight) was determined. The results are shown in Table 2. The results indicate the addition of yeast extract up to 0.8g/l of medium can increase the yield of cells. However, addition of corn steep liquor is even more effective and results in twice the yield of treatments with added yeast extract. This is very advantageous for the economic production of cells as corn steep liquor is much less expensive than yeast extract.

Table 2

Treatment (Amount Nutrient Supplement Added)	Ash-Free Dry Weight (mg/l)
2.0ml yeast ext.	4000
1.5ml yeast ext.	4420
1.0ml yeast ext.	4300
0.5ml yeast ext.	2780
0.25ml yeast ext.	2700
2.0ml yeast ext.	4420
1.5ml yeast ext. + 0.5ml CSL*	6560
1.0ml yeast ext. + 1.0ml CSL	6640
0.5ml yeast ext. + 1.5ml CSL	7200
2.0ml CSL	7590

*CSL = corn steep liquor

Example 5. Enhanced highly unsaturated fatty acid content of strains isolated by method in Example 1 compared to ATCC strains (previously known strains)

A battery of 151 newly isolated strains, selected according to the method described in Example 1, were sampled in late exponential phase growth and quantitatively analyzed for highly unsaturated fatty acid content by gas-liquid chromatography. All strains were grown either in M1 medium or liquid FFM medium, whichever gave highest yield of cells. Additionally, five previously isolated Thraustochytrium or Schizochytrium species were obtained from the American Type Culture Collection, representing all the strains which could be obtained in viable form from the collection. These strains were: *T. aureum* (ATCC No. 28211), *T. aureum* (ATCC No. 34304), *T. roseum* (ATCC No. 28210), *T. striatum* (ATCC No. 34473) and *S. aggregatum* (ATCC No. 28209). The strains all exhibited abbreviated growth in conventional media, and generally showed improved growth in media of the present invention, including M5 medium and FFM medium, Example 2. The fatty acids production of each of the known strains was measured as described, based upon the improved growth of the strains in media of the invention.

Fatty acid peaks were identified by the use of pure compounds of known structure. Quantitation, in terms of percent by weight of total fatty acids, was carried out by integrating the chromatographic peaks. Compounds identified were: palmitic acid (C16:0), C20:4w6 and C22:1 (which were

not resolved separately by the system employed), C20:5w3, C22:5w6, C22:5w3, and C22:6w3. The remainder, usually lower molecular weight fatty acids, were included in the combined category of "other fatty acids." Total omega-3 fatty acids were calculated as the sum of 20:5w3, 22:5w3 and 22:6w3. Total omega-6 fatty acids were calculated as the sum of the 20:4/22:1 peak and the 22:5w6 peak.

The results are shown in Tables 3-4 and illustrated in Figs. 2-4. From Table 3 it can be seen that large numbers of strains can be isolated by the method of the invention, and that large numbers of strains outperform the previously known strains by several important criteria. For example, 102 strains produced at least 7.8% by weight of total fatty acids C20:5w3, a higher percentage of that fatty acid than any previously known strain. Strains 23B (ATCC No. 20892) and 12B (ATCC No. 20890) are examples of such strains. Thirty (30) strains of the invention produced at least 68% by weight of total fatty acids as omega-3 fatty acids, more than any previously known strain. Strain 23B (ATCC No. 20892) is an example of such strains. Seventy-six (76) strains of the invention yielded not more than 10% by weight of total fatty acids as omega-6 fatty acids, considered undesirable components of the human diet, lower than any previously known

TABLE 3: LIST OF STRAINS AND COMPOSITIONS UNDER STANDARD SCREENING CONDITIONS

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
30.4%	2.8%	6.6%	3.2%	0.2%	8.3%	48.5%	15.1%	6.0%	21
22.9%	0.4%	2.3%	15.5%	0.5%	47.0%	11.5%	49.7%	15.9%	ATCC20889
14.9%	6.5%	12.0%	11.8%	0.4%	49.7%	4.7%	62.1%	18.3%	U40-2
40.3%	1.7%	3.8%	8.6%	0.0%	8.2%	37.4%	12.0%	10.2%	21B
20.7%	0.4%	7.8%	0.0%	0.0%	1.1%	70.1%	8.9%	0.4%	BG1
26.0%	5.7%	1.5%	9.7%	0.7%	9.7%	46.7%	11.9%	15.4%	56A
16.4%	1.4%	10.0%	1.9%	2.2%	46.4%	21.8%	58.6%	3.3%	11A-1
23.7%	3.3%	10.5%	1.9%	1.8%	29.9%	28.9%	42.2%	5.2%	4A-1
18.7%	6.9%	9.2%	11.9%	3.2%	25.2%	24.9%	37.5%	18.8%	17B
15.4%	4.2%	7.3%	9.5%	0.9%	51.2%	11.6%	59.3%	13.7%	ATCC20891
22.3%	3.9%	7.6%	23.5%	0.5%	22.1%	20.2%	30.2%	27.4%	S44
14.4%	2.3%	15.0%	18.4%	0.7%	43.8%	5.5%	59.4%	20.7%	U30
22.1%	7.8%	3.1%	12.7%	1.0%	14.9%	38.3%	19.0%	20.5%	59A
18.1%	2.3%	6.9%	9.1%	0.8%	52.2%	10.6%	59.9%	11.4%	U37-2
15.8%	3.9%	8.8%	11.6%	1.2%	53.3%	5.5%	63.3%	15.5%	S50W
23.7%	3.8%	6.3%	6.9%	0.6%	43.0%	15.6%	50.0%	10.7%	ATCC20891
10.0%	0.0%	0.0%	0.0%	0.0%	0.0%	90.0%	0.0%	0.0%	UX
16.6%	6.3%	11.9%	13.3%	1.7%	43.0%	7.3%	56.6%	19.5%	LW9
17.3%	2.3%	8.4%	11.4%	0.7%	53.6%	6.5%	62.6%	13.6%	C32-2
23.8%	1.2%	6.4%	2.5%	1.9%	34.4%	29.8%	42.6%	3.7%	5A-1
17.1%	5.2%	11.1%	7.6%	2.2%	27.2%	29.6%	40.4%	12.9%	BG1
25.4%	2.2%	9.6%	7.0%	1.1%	46.0%	8.8%	56.7%	9.1%	U3
16.9%	12.0%	6.6%	16.2%	0.4%	25.1%	22.8%	32.1%	28.2%	55B
26.3%	2.6%	8.6%	2.0%	2.5%	32.4%	25.5%	43.5%	4.6%	18A
19.4%	0.3%	9.8%	0.0%	0.3%	38.4%	31.7%	48.6%	0.3%	32B
16.0%	16.7%	8.6%	18.4%	0.0%	22.5%	17.7%	31.1%	35.1%	56B
18.6%	7.7%	11.4%	3.6%	4.3%	31.7%	22.7%	47.4%	11.2%	SX2
17.8%	4.4%	16.2%	6.4%	3.7%	33.6%	17.8%	53.5%	10.9%	53B
16.8%	2.7%	13.8%	20.5%	1.4%	39.3%	5.5%	54.4%	23.3%	S49
20.8%	8.0%	8.9%	6.4%	1.7%	33.9%	20.3%	44.5%	14.4%	S3
14.8%	0.3%	3.7%	3.9%	0.0%	69.9%	7.4%	73.6%	4.2%	3A-1
28.1%	5.2%	12.7%	3.2%	0.9%	20.9%	29.0%	34.5%	8.4%	15A
20.9%	0.7%	8.5%	1.0%	0.0%	35.8%	33.0%	44.3%	1.7%	9A-1
15.7%	10.2%	8.8%	13.4%	1.5%	23.9%	26.3%	34.3%	23.7%	51B
16.2%	11.2%	7.8%	16.4%	1.5%	20.4%	26.5%	29.7%	27.6%	8A-1
20.5%	5.5%	8.6%	4.8%	2.7%	28.7%	29.2%	40.0%	10.3%	13A-1
16.1%	13.6%	11.1%	16.0%	0.0%	28.4%	14.8%	39.4%	29.6%	24B-2
16.9%	7.3%	16.4%	6.1%	0.0%	40.8%	12.4%	57.2%	13.4%	24B-1
16.2%	0.0%	10.9%	1.0%	0.0%	56.5%	15.5%	67.4%	1.0%	3B
17.0%	0.0%	5.0%	2.3%	0.0%	73.4%	2.3%	78.3%	2.3%	SBG5
20.8%	4.5%	5.8%	3.8%	1.0%	22.7%	41.3%	29.5%	8.4%	16B
19.0%	14.0%	8.3%	18.9%	0.7%	23.9%	15.2%	32.9%	32.9%	6A-1
18.0%	0.3%	10.1%	0.0%	0.0%	48.9%	22.7%	59.0%	0.3%	33B

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
16.7%	5.5%	14.8%	8.5%	1.7%	31.8%	21.0%	48.3%	13.9%	B40
15.0%	1.0%	11.7%	2.1%	0.9%	62.3%	6.9%	74.9%	3.1%	28A
17.8%	18.5%	8.1%	20.5%	0.0%	22.1%	12.9%	30.2%	39.0%	43B
16.9%	0.0%	3.4%	2.7%	0.0%	61.2%	15.8%	64.6%	2.7%	1A-1
15.6%	2.7%	11.4%	10.9%	0.8%	53.7%	4.9%	65.9%	13.6%	U41-2
16.5%	0.7%	3.9%	3.9%	0.0%	68.4%	6.7%	72.2%	4.6%	56B
14.4%	0.9%	10.9%	2.5%	1.0%	66.4%	3.8%	78.3%	3.4%	46A
17.6%	0.0%	2.4%	3.3%	0.0%	66.3%	10.4%	68.7%	3.3%	15A-1
25.0%	0.0%	3.3%	0.0%	1.4%	53.2%	17.1%	57.9%	0.0%	13A
16.1%	13.4%	9.3%	13.6%	0.0%	32.3%	15.3%	41.6%	27.0%	37B
16.5%	9.1%	13.2%	6.7%	0.0%	38.9%	15.6%	52.1%	15.9%	43B
16.1%	12.4%	12.0%	15.7%	0.8%	30.5%	12.5%	43.3%	28.1%	17B
13.8%	0.8%	11.5%	2.8%	0.0%	67.0%	4.1%	78.6%	3.6%	27A
17.5%	18.6%	9.0%	19.5%	0.0%	21.7%	13.7%	30.7%	38.1%	46B
21.4%	1.4%	18.9%	0.0%	5.0%	43.5%	9.9%	67.3%	1.4%	ATCC20890
17.7%	0.0%	0.6%	4.4%	0.0%	68.2%	9.1%	68.8%	4.4%	5A
17.6%	16.0%	9.6%	18.8%	0.0%	25.6%	12.4%	35.2%	34.8%	28B-2
14.0%	0.9%	13.2%	1.6%	0.0%	64.7%	5.5%	77.9%	2.6%	27B
19.5%	2.9%	16.6%	1.1%	1.6%	30.2%	28.1%	48.5%	4.0%	49B
17.2%	0.7%	6.8%	2.7%	0.0%	63.0%	9.6%	69.8%	3.4%	18B
14.4%	3.5%	13.5%	26.0%	1.0%	37.2%	4.4%	51.6%	29.5%	S49-2
16.1%	2.2%	15.7%	21.6%	0.0%	36.7%	7.8%	52.4%	23.7%	20B
17.3%	4.7%	14.3%	7.2%	2.9%	30.2%	23.5%	47.3%	11.9%	8B
11.5%	3.3%	11.3%	6.5%	1.1%	59.9%	6.5%	72.2%	9.8%	13B
16.6%	0.7%	10.7%	1.6%	0.0%	59.7%	10.8%	70.4%	2.2%	26A
16.1%	3.3%	13.5%	23.8%	0.0%	38.7%	4.7%	52.2%	27.1%	S42
15.6%	0.6%	12.1%	0.0%	0.0%	60.2%	11.5%	72.3%	0.6%	35B
19.5%	0.0%	1.4%	3.4%	0.0%	66.6%	9.1%	68.0%	3.4%	42A
18.9%	3.5%	12.7%	25.0%	0.0%	35.0%	5.0%	47.6%	28.5%	40A
25.2%	3.3%	9.3%	21.8%	0.0%	30.3%	10.1%	39.6%	25.1%	S50C
17.6%	11.1%	13.2%	14.1%	1.3%	28.7%	14.0%	43.2%	25.2%	59A
19.9%	0.0%	5.5%	1.9%	0.0%	66.8%	6.0%	72.3%	1.9%	SBG9
15.4%	3.1%	13.2%	26.1%	0.0%	35.8%	6.5%	49.1%	29.1%	21B
18.9%	0.7%	11.6%	0.0%	0.0%	59.1%	9.7%	70.7%	0.7%	2B
14.1%	1.1%	12.4%	2.0%	0.0%	65.2%	5.2%	77.6%	3.1%	1B
22.2%	16.2%	6.3%	17.7%	0.0%	18.1%	19.5%	24.4%	33.8%	55B
16.0%	1.0%	4.5%	0.0%	0.0%	69.5%	9.0%	74.0%	1.0%	3A
17.0%	4.3%	12.4%	29.8%	0.0%	34.0%	2.5%	46.4%	34.1%	9B
15.4%	4.3%	8.7%	13.2%	0.0%	53.2%	5.1%	62.0%	17.5%	U24
14.2%	3.1%	12.0%	20.0%	1.1%	35.2%	14.3%	48.3%	23.2%	U28
16.8%	14.6%	10.1%	16.0%	0.6%	27.7%	14.0%	38.5%	30.7%	28B-1
23.2%	1.9%	8.3%	1.1%	2.3%	22.7%	40.4%	33.3%	3.0%	44B
24.6%	15.8%	8.7%	16.0%	0.0%	15.3%	19.6%	24.0%	31.8%	54B
15.5%	0.0%	1.3%	2.9%	0.0%	72.7%	7.6%	74.0%	2.9%	55A
18.4%	1.0%	5.0%	3.0%	0.0%	66.2%	6.4%	71.3%	3.9%	49A
18.6%	15.3%	9.4%	18.0%	0.0%	27.3%	11.4%	36.7%	33.3%	51A
23.5%	13.1%	7.3%	17.9%	0.0%	26.7%	11.4%	34.0%	31.0%	14A-1
13.3%	1.1%	14.5%	0.9%	0.0%	64.6%	5.6%	79.1%	2.0%	25B
22.9%	2.4%	10.3%	21.5%	0.0%	26.5%	16.4%	36.9%	23.9%	41A
16.8%	1.0%	9.7%	2.7%	0.0%	58.3%	11.5%	68.0%	3.7%	24A
0.4%	8.5%	14.1%	10.2%	2.1%	27.6%	37.0%	43.8%	18.8%	61A

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
30.5%	0.0%	7.1%	0.0%	0.0%	0.6%	61.8%	7.7%	0.0%	BRBG
18.2%	14.9%	8.3%	18.7%	0.0%	24.4%	15.5%	32.7%	33.6%	17A
17.4%	2.0%	9.3%	2.8%	0.0%	55.7%	12.7%	65.0%	4.9%	60A
14.1%	0.8%	13.0%	1.2%	0.0%	67.8%	3.1%	80.8%	2.0%	26B
17.8%	5.0%	6.9%	15.0%	1.5%	47.4%	6.4%	55.8%	20.0%	ATCC20888
16.0%	0.0%	1.8%	2.0%	0.0%	70.8%	9.4%	72.6%	2.0%	2A
24.6%	0.0%	4.0%	0.0%	0.0%	49.4%	22.0%	53.4%	0.0%	44A
17.4%	1.8%	0.0%	2.9%	0.0%	55.3%	23.3%	55.3%	4.6%	14A
23.3%	1.3%	4.6%	0.0%	0.0%	12.6%	58.1%	17.3%	1.3%	41B
19.3%	0.0%	1.1%	3.8%	0.0%	66.6%	9.1%	67.8%	3.8%	66A
18.6%	15.6%	8.3%	17.1%	1.1%	24.6%	14.8%	33.9%	32.7%	11A
19.6%	5.1%	10.1%	27.2%	0.0%	27.5%	10.6%	37.5%	32.3%	2X
15.7%	2.4%	14.0%	25.7%	0.0%	36.7%	5.4%	50.8%	28.1%	33A
14.6%	1.5%	13.5%	0.0%	0.0%	66.0%	4.3%	79.5%	1.5%	ATCC20892

PRIOR STRAINS

PER CENT OF TOTAL FATTY ACIDS							Total	Total	Strain
C16:0	C20:4w6	C20:5w3	C22:5w6	C22:5w3	C22:6w3	Other FA	Omega3	Omega6	
15.7%	3.9%	3.7%	8.1%	0.0%	55.1%	13.5%	58.8%	12.0%	ATCC34304
28.2%	1.6%	6.9%	11.4%	0.0%	17.8%	34.1%	24.7%	12.9%	ATCC24473
15.2%	2.9%	7.7%	9.8%	0.6%	54.6%	9.2%	62.9%	12.7%	ATCC28211
23.2%	10.7%	4.3%	12.6%	1.5%	20.6%	27.0%	26.4%	23.4%	ATCC28209
13.2%	6.3%	6.9%	4.3%	0.0%	60.1%	9.1%	67.0%	10.6%	ATCC28210

TABLE 4: COMPOSITION OF OMEGA 3 FATTY ACID FRACTION

EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
44.0%	1.1%	54.9%	21
4.6%	0.9%	94.5%	ATCC20889
19.3%	0.7%	80.0%	U40-2
31.9%	0.0%	68.1%	21B
87.9%	0.0%	12.1%	BRBG1
12.5%	6.1%	81.5%	56A
17.0%	3.7%	79.3%	11A-1
24.9%	4.3%	70.8%	4A-1
24.4%	8.4%	67.2%	17B
12.2%	1.5%	86.3%	ATCC20891
25.1%	1.7%	73.2%	S44
25.2%	1.1%	73.7%	U30
16.2%	5.4%	78.4%	59A
11.5%	1.4%	87.1%	U37-2
14.0%	1.9%	84.2%	S50W
12.7%	1.3%	86.0%	ATCC20891
---	---	---	UX
21.0%	2.9%	76.1%	LWN9
13.4%	1.0%	85.6%	C32-2
15.0%	4.3%	80.7%	5A-1
27.4%	5.4%	67.2%	BRBG1
17.0%	1.9%	81.1%	U3
20.5%	1.3%	78.2%	55B
19.8%	5.8%	74.4%	18A
20.1%	0.7%	79.2%	32B
27.8%	0.0%	72.2%	56B
24.1%	9.1%	66.9%	SX2
30.3%	6.9%	62.8%	53B
25.3%	2.5%	72.2%	S49
19.9%	3.8%	76.3%	S3
5.0%	0.0%	95.0%	3A-1
36.9%	2.6%	60.5%	15A
19.3%	0.0%	80.7%	9A-1
25.8%	4.4%	69.8%	51B
26.3%	5.0%	68.7%	8A-1
21.6%	6.7%	71.7%	13A-1
28.0%	0.0%	72.0%	24B-2
28.7%	0.0%	71.3%	24B-1
16.2%	0.0%	83.8%	3B
6.3%	0.0%	93.7%	SBG5
19.7%	3.3%	77.0%	16B
25.2%	2.1%	72.6%	6A-1
17.1%	0.0%	82.9%	33B
30.5%	3.6%	65.9%	B40
15.6%	1.2%	83.1%	28A

EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
26.8%	0.0%	73.2%	43B
5.2%	0.0%	94.8%	1A-1
17.4%	1.2%	81.5%	U41-2
5.4%	0.0%	94.6%	56B
13.9%	1.3%	84.8%	46A
3.5%	0.0%	96.5%	15A-1
5.8%	2.4%	91.8%	13A
22.3%	0.0%	77.7%	37B
25.4%	0.0%	74.6%	43B
27.7%	1.9%	70.3%	17B
14.7%	0.0%	85.3%	27A
29.2%	0.0%	70.8%	46B
28.0%	7.5%	64.5%	ATCC20890
0.9%	0.0%	99.1%	5A
27.3%	0.0%	72.7%	28B-2
16.9%	0.0%	83.1%	27B
34.3%	3.4%	62.3%	49B
9.7%	0.0%	90.3%	18B
26.1%	1.9%	71.9%	S49-2
29.9%	0.0%	70.1%	20B
30.1%	6.2%	63.7%	8B
15.6%	1.5%	82.9%	13B
15.2%	0.0%	84.8%	26A
25.9%	0.0%	74.1%	S42
16.7%	0.0%	83.3%	35B
2.1%	0.0%	97.9%	42A
26.6%	0.0%	73.4%	40A
23.4%	0.0%	76.6%	S50C
30.6%	2.9%	66.4%	59A
7.6%	0.0%	92.4%	SBG9
27.0%	0.0%	73.0%	21B
16.4%	0.0%	83.6%	2B
15.9%	0.0%	84.1%	1B
25.9%	0.0%	74.1%	55B
6.0%	0.0%	94.0%	3A
26.7%	0.0%	73.3%	9B
14.1%	0.0%	85.9%	U24
24.9%	2.2%	72.9%	U28
26.4%	1.5%	72.1%	28B-1
24.8%	6.9%	68.3%	44B
36.4%	0.0%	63.6%	54B
1.8%	0.0%	98.2%	55A
7.1%	0.0%	92.9%	49A
25.6%	0.0%	74.4%	51A
21.5%	0.0%	78.5%	14A-1
18.4%	0.0%	81.6%	25B
28.1%	0.0%	71.9%	41A
14.3%	0.0%	85.7%	24A
32.3%	4.8%	63.0%	61A
91.6%	0.0%	8.4%	BRBG

EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
25.5%	0.0%	74.5%	17A
14.4%	0.0%	85.6%	60A
16.1%	0.0%	83.9%	26B
12.4%	2.7%	84.9%	ATCC20888
2.5%	0.0%	97.5%	2A
7.5%	0.0%	92.5%	44A
0.0%	0.0%	100.0%	14A
26.7%	0.0%	73.3%	41B
1.7%	0.0%	98.3%	66A
24.5%	3.1%	72.4%	11A
26.8%	0.0%	73.2%	2X
27.6%	0.0%	72.4%	33A
17.0%	0.0%	83.0%	ATCC20892

PRIOR STRAINS

EPA C20:5w3	DPA C22:5w3	DHA C22:6w3	Strain
6.4%	0.0%	93.6%	ATCC34304
27.9%	0.0%	72.1%	ATCC24473
12.2%	1.0%	86.8%	ATCC28211
16.4%	5.6%	78.1%	ATCC28209
10.3%	0.0%	89.7%	ATCC28210

strain. Strains 23B (ATCC No. 20892) and 12B (ATCC No. 20890) are examples of such strains. In addition, there are 35 strains of the invention that produce more than 25% by weight of total fatty acids as omega-6 fatty acids, more than any previously known strain. While such strains may not be useful for dietary purposes, they are useful as feedstock for chemical synthesis of eicosanoids starting from omega-6 fatty acids.

In addition, the data reveal many strains of the invention which produce a high proportion of total omega-3 fatty acids as C22:6w3. In Table 4, 48 of the strains shown in Table 2 were compared to the previously known strains, showing each of C20:5w3, C22:5w3 and C22:6w3 as percent by weight of total omega-3 content. Fifteen strains had at least 94% by weight of total omega-3 fatty acids as C22:6w3, more than any previously known strain. Strain S8 (ATCC No. 20889) was an example of such strains. Eighteen strains had at least 28% by weight of total omega-3 fatty acids as C20:5w3, more than any previously known strain. Strain 12B (ATCC No. 20890) was an example of such strains.

Figure 2 illustrates the set of strains, isolated by the method in Example 1, that have more than 67% omega-3 fatty acids (as % of total fatty acids) and less than 10.6% omega-6 fatty acids (as % of total fatty acids). All of the previously known strains had less than 67% omega-3 fatty acids

(as % of total fatty acids) and greater than 10.6% omega-6 (as % of total fatty acids).

Figure 3 illustrates the set of strains, isolated by the method in Example 1, that have more than 67% omega-3 fatty acids (as % of total fatty acids) and greater than 7.5% C20:5w3 (as % of total fatty acids). All of the previously known strains had less than 67% omega-3 fatty acids (as % of total fatty acids) and less than 7.8% C20:5w3 (as % of total fatty acids).

Example 6. Enhanced growth rates of strains isolated by method in Example 1 compared to ATCC strains (previously known strains)

Cells of Schizochytrium sp. S31 (ATCC No. 20888), Schizochytrium sp. S8 (ATCC No. 20889), Thraustochytrium sp. S42, Thraustochytrium sp. U42-2, Thraustochytrium sp. S42 and U30, (all isolated by the method of Example 1) and Thraustochytrium aureum (ATCC #28211) and Schizochytrium aggregatum (ATCC #28209) (previously known strains) were picked from solid F-1 medium and placed into 50ml of M-5 medium. This medium consists of (on a per liter basis): Yeast Extract, 1g; NaCl, 25g; $MgSO_4 \cdot 7H_2O$, 5g; KCl, 1g; $CaCl_2$, 200mg; glucose, 5g; glutamate, 5g; KH_2PO_4, 1g; PII metals, 5ml; A-vitamins solution, 1ml; and antibiotic solution, 1ml. The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. After three days of growth on an orbital shaker (200 rpm, 27°C), 1-2ml of each culture was

transferred to another flask of M-5 medium and placed on the shaker for 2 days. The cultures (1-2ml) were then transferred to another flask of M-5 medium and placed on the shaker for 1 day. This process ensured that all cultures were in the exponential phase of growth. These later cultures were then used to inoculate two 250ml flasks of M-5 medium for each strain. These flasks were then placed on shakers at 25°C and 30°C, and changes in their optical density were monitored on a Beckman DB-G spectrophotometer (660nm, 1cm path length). Optical density readings were taken at the following times: 0, 6, 10, 14, 17.25, 20.25 and 22.75 hours. Exponential growth rates (doublings/day) were then calculated from the optical density data by the method of Sorokin (1973). The results are presented in Table 5 and illustrated (normalized to the growth of strain U30 at 25°C) in Fig. 5. The data indicate that the strains isolated by the method in Example 1 have much higher growth rates than the previously known ATCC strains at both 25°C and 30°C, even under the optimized phosphate levels essential for continuous growth. Strains of Thraustochytriales isolated from cold Antarctic waters have not been shown to grow at 30°C.

Table 5

Exponential Growth Rate
(doublings/day)

Strain	25°C	30°C
S31*	8.5	9.4
U40-2*	5.8	6.0
S8*	7.1	8.8
S42*	6.6	8.3
U30*	5.5	7.3
28209**	4.6	5.0
28210**	3.5	4.5
28211**	4.2	5.7
34304**	2.7	3.7
24473**	4.6	5.3

* strain isolated by method in Example 1

** previously known ATCC strain

Example 7. Enhanced production characteristics (growth and lipid induction) of strains isolated by method in Example 1 compared to ATCC strains (prior art strains)

Cells of Schizochytrium sp. S31 (ATCC No. 20888), Schizochytrium sp. S8 (ATCC No. 20889) (both isolated by the method of Example 1) and Thraustochytrium aureum (ATCC #28211) and Schizochytrium aggregatum (ATCC #28209) (prior art strains) were picked from solid F-1 medium and placed into 50ml of M-5 medium (see Example 5). The pH of the solution was adjusted to 7.0 and the solution was filter sterilized. After three days of growth on an orbital shaker (200 rpm, 27°C), 1-2ml of each culture was transferred to another flask of M-5 medium and placed on the shaker for 2 days. The ash-free dry weights for each of these cultures were then quickly determined and 3.29mg of each culture was pipetted into two 250ml erlenmeyer flasks containing 50ml of M-5 medium. These flasks were placed on a rotary shaker (200 rpm, 27°C). After 24 hours 20ml portions of each culture were then centrifuged, the supernatants discarded, and the cells transferred to 250ml erlenmeyer flasks containing 50 ml of M-5 medium without any glutamate (N-source). The flasks were placed back on the shaker, and after another 12 hours they were sampled to determine ash-free dry weights and quantify fatty acid contents by the method of Lepage and Roy (1984). The results are illustrated (normalized to the yields of ATCC No. 28211, previously known strain) in Fig. 6. The results indicate that the strains isolated by the method of Example 1 produced 2-3

times as much ash-free dry weight in the same period of time, under a combination of exponential growth and nitrogen limitation (for lipid induction) as the prior art ATCC strains. In addition, higher yields of total fatty acids and omega-3 fatty acids were obtained from strains of the present invention with strains S31 (ATCC No. 20888) producing 3-4 times as much omega-3 fatty acids as the prior art ATCC strains.

Example 8. Enhanced salinity tolerance and fatty acid production by strains isolated by method in Example 1

Strains of 4 species of Oomycetes, *Schizochytrium* sp. S31 (ATCC No. 20888) and *Thraustochytrium* sp. U42-2 (ATCC No. 20891) (both isolated and screened by the method of Example 1), and *S. aggregatum* (ATCC 28209) and *T. aureum* (ATCC 28210) (obtained from the American Type Culture Collection) were picked from solid F-1 medium and incubated for 3-4 days at 27°C on a rotary shaker (200 rpm). A range of differing salinity medium was prepared by making the following dilutions of M medium salts (NaCl, 25g/l; $MgSO_4 \cdot 7H_2O$, 5g/l; KCl, 1g/l; $CaCl_2$, 200mg/l): 1) 100% (w/v M medium salts; 2) 80% (v/v) M medium, 20% (v/v) distilled water; 3) 60% (v/v) M medium, 40% (v/v) distilled water; 4) 40% (v/v) M medium, 60% (v/v) distilled water; 5) 20%(v/v) M medium, 80% distilled water; 6) 15% (v/v) M medium, 85% (v/v) distilled water; 7) 10% (v/v) M medium, 90% (v/v) distilled water; 8) 7% (v/v) M medium, 93% (v/v) distilled water; 9) 3% (v/v) M medium, 97% (v/v)

distilled water; 10) 1.5% (v/v) M medium, 98.5% (v/v) distilled water. The following nutrients were added to the treatments (per liter): glucose, 5g; glutamate, 5g; yeast ext., 1g; $(NH_4)_2SO_4$, 200 mg; $NaHCO_3$, 200 mg; PII metals, 5ml; A-vitamins solution, 1ml; and antibiotics solution, 2ml. Fifty ml of each of these treatments were inoculated with 1ml of the cells growing in the F-1 medium. These cultures were placed on an orbital shaker (200 rpm) and maintained at 27°C for 48 hr. The cells were harvested by centrifugation and total fatty acids determined by gas chromatography. The results are illustrated in Fig. 7. Thraustochytrium sp. U42-2 (ATCC No. 20891) isolated by the method of Example 1 can yield almost twice the amount of fatty acids produced by T. aureum (ATCC 28210) and over 8 times the amount of fatty acids produced by S. aggregatum (ATCC 28209). Additionally, U42-2 appears to have a wider salinity tolerance at the upper end of the salinity range evaluated. Schizochytrium sp. S31 (ATCC No. 20888), also isolated by the method in Example 1, exhibited both a high fatty acid yield (2.5 to 10 times that of the previously known ATCC strains) and a much wider range of salinity tolerance than the ATCC strains. Additionally, Schizochytrium sp. S31 (ATCC No. 20888) grows best at very low salinities. This property provides a strong economic advantage when considering commercial production, both because of the corrosive effects of saline waters on metal reactors, and because of problems associated with the disposal of saline waters.

Example 9. Cultivation/Low Salinity

Fifty ml of M/10-5 culture media in a 250ml erlenmeyer flask was inoculated with a colony of Schizochytrium sp. S31 (ATCC No. 20888) picked from an agar slant. The M/10-5 media contains: 1000ml deionized water, 2.5g NaCl, 0.5g $MgSO_4 \cdot 7H_2O$, 0.1g KCl, 0.02g $CaCl_2$, 1.0g KH_2PO_4, 1.0g yeast extract, 5.0g glucose, 5.0g glutamic acids, 0.2g $NaHCO_3$, 5ml PII trace metals, 2ml vitamin mix, and 2ml antibiotic mix. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. 20ml of this actively growing culture was used to inoculate a 2 liter fermenter containing 1700ml of the same culture media except the concentration of the glucose and glutamate had been increased to 40g/l (M/10-40 media). The fermenter was maintained at 30°C, with aeration at 1 vol/vol/min, and mixing at 300 rpm. After 48 hr, the concentration of cells in the fermenter was 21.7g/l. The cells were harvested by centrifugation, lyophilized, and stored under N_2.

The total fatty acid content and omega-3 fatty acid content was determined by gas chromatography. The total fatty acid content of the final product was 39.0% ash-free dry weight. The omega-3 highly unsaturated fatty acid content (C20:5w3, C22:5w3 and C22:6w3) of the microbial product was 25.6% of the ash-free dry weight. The ash content of the sample was 7.0%.

Example 10.

Growth and gas chromatographic analysis of fatty acid production by various strains as described in example 5 revealed difference in fatty acid diversity. Strains of the present invention synthesized fewer different fatty acids than previously available strains. Lower diversity of fatty acids is advantageous in fatty acid purification since there are fewer impurities to be separated. For food supplement purposes, fewer different fatty acids is advantageous because the likelihood of ingesting unwanted fatty acids. Table 6 shows the number of different highly unsaturated fatty acids present, at concentrations greater than 1% by weight of total fatty acids for previously known strains, designated by ATCC number and various strains of the present invention.

Table 6

Strain	No. of Different Fatty Acids at 1% or Greater % of Total Fatty Acids
34304**	8
28211**	8
24473**	10
28209**	13
28210**	8
S31*	5
S8*	6
79B*	6

* strain isolated by the method in Example 1

** previously known ATCC strain

Example 11. Recovery

Fifty ml of M5 culture media in a 250 ml erlenmeyer flask was inoculated with a colony of Schizochytrium sp. S31 (ATCC No. 20888) picked from an agar slant. The M5 media contains: 1000ml deionized water, 25.0g NaCl, 5.0g $MgSO4 \cdot 7H_2O$, 1.0g KCl, 0.2g $CaCl_2$, 1.0g KH_2PO_4, 1.0g yeast extract, 5.0g glucose, 5.0g glutamic acid, 0.2g $NaHCO_3$, 5ml PII trace metals, 2ml vitamin mix, and 2ml antibiotic mix. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. 20ml of this actively growing culture was used to inoculate an 1 liter fermenter containing 1000ml of the same culture media except the concentration of the glucose and glutamate had been increased to 40g/l (M20 media). The fermenter was maintained at 30°C and pH 7.4, with aeration at 1 vol/min, and mixing at 400 rpm. After 48 hr, the concentration of the cells in the fermenter was 18.5g/l. Aeration and mixing in the fermenter was turned off. Within 2-4 minutes, the cells flocculated and settled in the bottom 250 ml of the fermenter. This concentrated zone of cells had a cell concentration of 72g/l. This zone of cells can be siphoned from the fermenter, and: (1) transferred to another reactor for a period of nitrogen limitation (e.g., combining the highly concentrated production of several fermenters); or (2) harvested directly by centrifugation or filtration. By preconcentrating the cells in this manner, 60-80% less water has to be processed to recover the cells.

Example 12. Utilization of a variety of carbon and nitrogen sources.

Fifty ml of M5 culture media in a 250ml erlenmeyer flask was inoculated with a colony of Schizochytrium sp. S31 (ATCC No. 20888) or Thraustochytrium sp. U42-2 (ATCC No. 20891) picked from an agar slant. The M5 media was described in Example 4 except for 2ml vitamin mix, and 2ml antibiotic mix. The culture was incubated at 30°C on a rotary shaker (200 rpm). After 2 days the culture was at a moderate density and actively growing. This culture was used to inoculate flasks of M5 media with one of the following substituted for the glucose (at 5g/l): dextrin, sorbitol, fructose, lactose, maltose, sucrose, corn starch, wheat starch, potato starch, ground corn; or one of the following substituted for the glutamate (at 5g/l): gelysate, peptone, tryptone, casein, corn steep liquor, urea, nitrate, ammonium, whey, or corn gluten meal. The cultures were incubated for 48 hours on a rotary shaker (200 rpm, 27°C). The relative culture densities, representing growth on the different organic substrates, are illustrated in Tables 7-8.

Table 7. Utilization of Nitrogen Sources

N-Source	Strains	
	Thraustochytrium sp. U42-2 ATCC No. 20891	_Schizochytrium_ sp. S31 ATCC No. 20888
glutamate	+++	+++
gelysate	+++	+++
peptone	++	++
tryptone	++	++
casein	++	++
corn steep liquor	+++	+++
urea	+	++
nitrate	++	+++
ammonium	+	+++
whey	+++	+++
corn gluten meal	+++	+++

+++ = high growth
++ = medium growth
+ = low growth
0 = no growth

Table 8. Utilization of Organic Carbon Sources.

C-Source	Strains *Thraustochytrium* sp. U42-2 ATCC No. 20891	*Schizochytrium* sp. S31 ATCC No. 20888
glucose	+++	+++
dextrin	+++	+++
sorbitol	+	+
fructose	+	+++
lactose	+	+
maltose	+++	+
sucrose	+	+
corn starch	+++	+
wheat starch	+++	+
potato starch	+++	+
ground corn	+++	0

+++ = high growth
++ = medium growth
+ = low growth
0 = no growth

Example 13: Feeding of thraustochytrid-based feed supplement to brine shrimp to increase their omega-3 HUFA content

Cellular biomass of Thraustochytrium sp. 12B (ATCC 20890) was produced in shake flasks in M-5 medium (see Example 6) at 25°C. Cellular biomass of Thraustochytrium sp. S31 (ATCC 20888) was produced in shake flasks in M-5/10 medium (see Example 9) at 27°C. The cells of each strain were harvested by centrifugation. The pellet was washed once with distilled water and recentrifuged to produce a 50% solids paste. The resulting paste was resuspended in sea water and then added to an adult brine shrimp culture as a feed supplement. The brine shrimp had previously been reared on agricultural waste products and as a result their omega-3 HUFA content was very low, only 1.3 - 2.3% of total fatty acids (wild-caught brine shrimp have an average omega-3 HUFA content of 6 - 8% total fatty acids). The brine shrimp (2 - 3/mL) were held in a 1 liter beaker filled with sea water and an airstone was utilized to aerate and mix the culture. After addition of the feed supplement, samples of the brine shrimp were periodically harvested, washed, and their fatty acid content determined by gas chromatography. The results are illustrated in Figs. 8 - 9. When fed the thraustochytrid-based feed supplement as a finishing feed, the omega-3 content of the brine shrimp can be raised to that of wild-type brine shrimp within 5 hours if fed strain 12B or within 11 hours when fed strain S31. The omega-3 HUFA content of the brine shrimp can be greatly enhanced over that of the wild type if

fed these feed supplements for up to 24 hours. Additionally, these feed supplements greatly increase the DHA content of the brine shrimp, which is generally only reported in trace levels in wild-caught brine shrimp.

Example 14: Feeding of thraustochytrid-based feed supplement to laying hens to produce omega-3 HUFA enriched eggs

NEW

Cellular biomass of Thraustochytrium sp. S31 (ATCC 20888) was produced in a 10 liter fermenter in M-5/10 medium (see Example 9) at 27°C. The cells of Thraustochytrium sp. S31 (ATCC 20888) were harvested by centrifugation, washed once with distilled water and recentrifuged to produce a 50% solids paste. This cell paste was then treated in one of two ways: 1) lyophilized; or 2) mixed with ground corn to produce a 70% solids paste and then extruded at 90 - 120°C and air dried. The resulting dried products were then ground, analyzed for omega-3 HUFA content, and mixed into layers rations at a level to provide 400 mg of omega-3 HUFA per day to the laying hens (400 mg omega-3 HUFA/100 grams layers ration). The resulting eggs were sampled over a period of approximately 45 days and analyzed by gas chromatography for omega-3 HUFA's. Eggs with up to 200 - 425 mg omega-3 HUFA's/egg were produced by the hen fed omega-3 supplement. When cooked, these eggs did not exhibit any fishy odors. The control hens produced eggs with only approximately 20 mg omega-3 HUFA/egg. There was no significant difference between the number of eggs laid by the control group and the hen fed the omega-3 supplement. There

was also no difference in the color of yolks of the eggs produced with the feed supplement and the control diet.

EXAMPLE 15: Production of high purity (>90% purity omega-3 HUFA or >90% purity HUFA fatty acids mixtures)

NEW

Cellular biomass of Thraustochytrium sp. S31 (ATCC 20888) was produced in a 10 liter fermenter in M-5/10 medium (see Example 9) at 27°C. The cells of this strain were harvested by centrifugation. Approximately 5 g of the cell paste was placed in the 350 mL stainless steel grinding chamber of a Bead-Beater bead mill which was filled 1/2 way with 0.5 mm glass beads. The remaining volumes of the chamber was filled with reagent grade MeOH and the cells homogenized for two 3 minute periods. During the bead mill operation, the stainless steel chamber was kept cold by an attached ice bath. The solution of broken cells was poured into a flask to which was added both chloroform and a 2M NaCl solution in water to bring the final solution to approximately 1:1:0.9 (chloroform:MeOH:water). The solution was then poured into a separatory funnel and shaken several times to help move the lipids into the chloroform fraction. After the solution was allowed to settle for several minutes, the chloroform fraction was collected into a flask, another portion of fresh chloroform added to the separatory funnel and the extraction repeated. This fraction of chloroform was then collected from the separatory funnel and the two chloroform portions combined. The chloroform was then removed (and recovered) by

using a roto-vap rotary vacuum evaporation device operated at 40°C. A portion (300mg) of the remaining lipids was removed and hydrolyzed for 6 hours at 60°C (under nitrogen gas) in 50 mL of solution of methanolic NaOH (10 mL of 0.3 N NaOH diluted to 100mL with MeOH) in a 150 mL teflon lined screw capped bottle. The nonsaponifiable materials (sterols, hydrocarbons, etc.) were then removed by phase separating the solution with two 50 mL portions of petroleum ether in a separatory funnel, discarding the ether fraction each time. The remaining solution was then acidified by addition of 3 mL of 6 N HCl and the free fatty acids extracted with two 50 mL portions of petroleum ether. Five mL portion of the ether solution containing the free fatty acids was placed in three 13mm X 100mm test tubes and the ether removed by blowing down the solution under a flow of nitrogen gas. Two mL portions of either petroleum ether, hexane or acetone were then added to one of tubes, which was then caped and placed in a solution of dry ice and ethanol (-72 to -74°C) to allow the non-HUFA fatty acids to crystallize. When crystallization appeared complete, the culture tubes were placed in 50 mL polycarbonate centrifuge tubes that had been filled with finely powdered dry ice. These tubes were then placed in a refrigerated centrifuge at -10°C and centrifuged for 3-5 minutes to 10,000 rpm. The supernatant was then quickly removed from each tube with a pasteur pipet and placed in a clean culture tube. The solvent was removed from the supernatants by blowing down under N_2. The fatty acids were then methylated in methanolic H_2SO_4 (4 mL H_2SO_4 in 96 mL MeoH) at 100°C for 1 hr in teflon

lined, screw capped tubes under N_2. The fatty acid methyl esters were then quantified by gas chromatography (HP 5890 gas chromatograph, Supelco SP 2330 column; column temp = 200°C; detector and injector temp = 250°C; carrier gas = nitrogen). The composition of the fatty acid mixtures obtained were: (ether) 93.1% HUFA's - 23.4% C22:5n-6 + 69.7% 22:6n-3; (hexane) 91.5% HUFA's - 66.8% 22:6n-3 + 22.1% 22:5n-6 + 2.6% 20:5n-3; (acetone) 90.0% HUFA's - 65.6% 22:6n-3 + 21.8n-6 + 2.6% 20:5n-3.

New

A fatty acid mixture containing >90% omega-3 HUFA's can be obtained by running the above process on harvested biomass of a strain of thraustochytrid such as 12B (ATCC 20890).

General Concluding Remarks

The following novel strains, isolated according to the method of the invention, were placed on deposit at the American Type Culture Collection (ATCC), Rockville, MD, as exemplars of the organisms disclosed and claimed herein.

Strain	ATCC No.	Deposit Date
Schizochytrium S31	20888	8/8/88
Schizochytrium S8	20889	8/8/88
Schizochytrium 12B	20890	8/8/88
Thraustochytrium U42-2	20891	8/8/88
Schizochytrium 23B	20892	8/8/88

The present invention, while disclosed in terms of specific organism strains, is intended to include all such methods and

strains obtainable and useful according to the teachings disclosed herein, including all such substitutions, modification, and optimizations as would be available expedients to those of ordinary skill in the art.

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